A Field Evaluation of an Indirect Immunofluorescent Antibody Test Developed to Diagnose Plasmacytoid Leukemia in Chinook Salmon (*Oncorhynchus tshawytscha*)

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ABSTRACT

An immunofluorescent antibody test (IFAT) developed for the diagnosis for plasmacytoid leukemia was evaluated against histology under field conditions. Previously published results from a laboratory evaluation indicated that the IFAT had a much higher sensitivity than did histology. One hundred seventy-seven moribund chinook salmon from 3 farms located in British Columbia were sampled. Sensitivity, specificity and their respective quality indices were estimated for the IFAT relative to histology. The IFAT was shown to be unreliable, particularly with respect to sensitivity. Cohen's kappa was also calculated and revealed that the agreement between the 2 tests was no better than random. In contrast to previously published results the IFAT did not perform better than histology in the presence of bacterial kidney disease. The results emphasize the importance of evaluating tests in the field conditions in which they are to be used. The possible reasons for the shortcomings of the IFAT are discussed.

RÉSUMÉ

Une épreuve d'immunofluorescence indirecte (IFI) mise au point pour le diagnostic de la leucémie plasmocytaire a été comparée à l'examen histologique sous des conditions de champs. Des résultats préliminaires obtenus lors d'une évaluation en laboratoire démontraient que l'épreuve IFI avait une plus grande sensibilité que l'examen histologique. Un total de 177 saumons chinook provenant de trois fermes situées en Colombie-Britannique ont été échantillonnés. La sensibilité et la spécificité de même que leur indice de qualité respectif ont été estimés pour l'IFI par rapport à l'examen histologique. Il a été démontré que l'IFI n'était pas fiable, particulièrement en ce qui a trait à la sensibilité. Le test kappa de Cohen a également été calculé et a démontré que la corrélation entre les deux épreuves n'était pas meilleure que pour des valeurs prises au hasard. Contrairement aux résultats déjà publiés, l'épreuve IFI n'était pas meilleure que l'examen histologique lorsqu'en présence de maladie bactérienne du rein. Les résultats démontrent l'importance d'évaluer les épreuves diagnostiques dans des conditions similaires à celles dans lesquelles elles seront utilisées.

(Traduit par le docteur Serge Messier)

INTRODUCTION

Plasmacytoid leukemia (PL), commonly known as marine anemia, is a disease of farmed chinook salmon (*Oncorhynchus tshawytscha*) in British Columbia (BC) (1). The disease is characterized grossly by pale gills, splenomegaly, renomegaly and exophthalmia (2). Mortality rates attributed to PL have been reported to range from 2.5% to 20% (1,3,4). A survey performed by Stephen et al (4) found the disease to be an endemic problem.

Both a retrovirus and the microsporidian, *Nucleospora salmonis*, have been proposed as possible etiologic agents (2,5–7). Until recently, diagnosis could only be made through histopathological evaluation. For a chinook to be diagnosed histologically with PL, large immature cells, referred to as plasmablasts, must be observed in massive numbers in the posterior kidney and one non-hematopoietic organ (1,6). Laboratory trials on experimentally infected fish determined that histological diagnosis for PL had a specificity of 100% (ie, no non-infected fish tested PL-positive with histology), which results in a 100% positive predictive value (8). This would indicate that all fish that test positive by histology have PL. The same trials, however, found that sensitivity was only 32% (ie, 32% of the PL-infected fish tested positive with histology). A low sensitivity would result in a large proportion of experimentally infected fish being misdiagnosed as PL-negative with histology. Histology has also been determined to be quite ineffective in diagnosing PL when fish have concurrent infections with *Renibacterium salmoninarum*, the causal agent of bacterial kidney disease (BKD) (2,8). BKD is a common disease of chinook salmon, which can cause extreme granulomatous reactions in many organs, particularly the kidney (9). In one study, BKD was diagnosed in 70% of moribund chinook salmon examined in BC (3,10). Histological changes attributed to PL might, therefore, be obstructed by the pathological changes of BKD.

In an attempt to improve PL diagnosis, an indirect immunofluorescent

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antibody test (IFAT) was developed with monoclonal antibodies (MAbs) from spleen cells of mice that had been immunized with retrobulbar and anterior kidney tissue preparations from naturally and experimentally infected chinook salmon that had been diagnosed PL-positive by histology (8,11). In laboratory trials, the IFAT was compared to histological diagnosis on fish that were experimentally infected via intraperitoneal injection of kidney and spleen homogenate collected from donar fish diagnosed PL-positive by histology. Using injection status as the gold standard, the IFAT was determined to have a sensitivity of 72% and a specificity of 76% (8). It was concluded that the IFAT was a more effective test for diagnosing PL, especially in the presence of BKD (8).

The next step in the development of the IFAT was to perform a field trial. Field trials have been incorporated in the development of several tests designed for fish disease diagnosis (12–14). The present study was carried out to evaluate the IFAT, in field conditions, on naturally infected production fish. Samples were collected between July and October, the time when PL appears to be most prevalent (4,15). Moribund fish were tested for the presence of PL using histology and IFAT. The agreement for the test was evaluated using kappa and sensitivity and specificity. For a test to be considered useful, sensitivity and specificity values both need to be high (16). Quality indices for the sensitivity and specificity of the IFAT, relative to histology, were also calculated. These indices relate the sensitivity and specificity to the apparent prevalence of the disease. Quality indices are similar to weighted kappas and have been suggested as a method to measure the strength of the sensitivity or specificity of legitimate tests (16). Therefore, a good test has quality indices that approach the value of one.

MATERIALS AND METHODS

SAMPLE COLLECTION

Samples were collected from 2-year-old chinook salmon at 3 chinook salmon seawater net-pen sites located in 3 different salmon farming regions of BC. Farm 1, located on the northern coast of Vancouver Island was visited once in July and twice in August, and a total of 97 fish were sampled. Farm 2, located on the west coast of Vancouver Island, was visited once in September, at which time 50 fish were sampled. Farm 3, located on the Sunshine coast was visited twice in September, and a total of 30 fish were sampled.

Slow-swimming, moribund salmon were caught by either dip-net or by a diver. Fish were killed by applying a blow to the cranium. Fish were immediately necropsied and examined grossly. Samples of posterior kidney, spleen, liver, heart, posterior intestine, pyloric ceca, and gill were collected and preserved in Davidson's fixative solution for histological processing (17).

The protocol used to collect samples for the IFAT was that outlined by Newbound et al (8). Small sections of anterior kidney (approximately 0.5 cm^2) and spleen were dissected out from each fish sampled. Excess blood was blotted off on a paper towel and tissue imprints were made onto a 12-well multi-spot microscopic slide (John's Scientific, Dartmouth, Nova Scotia). Kidney imprints were made on the top 6 wells and spleen imprints made on the bottom 6 wells. Two slides were made for each fish. The slides were allowed to air dry, and were then fixed in an acetone- 99% ethanol solution (60:40) and stored on ice for approximately 20 min. The slides were again allowed to air dry before being stored in a waterproof container and placed in an ice cooler for transportation back to the laboratory. Upon return to the laboratory, the slides were stored in a freezer at −20°C, with a desiccant, until the day of reading. All the laboratory work was performed at the Pacific Biological Station in Nanaimo, BC.

HISTOLOGY

Fixed tissues were prepared using standard histological procedures, and were stained with hematoxylin and eosin. The senior author examined one slide for each of the 177 fish sampled in order to assign a positive or negative PL diagnosis. A positive diagnosis for PL required presence of an increased number of plasmablast cells in both the renal interstitum and one other non-hematopoietic organ (8). Slides were also examined for evidence of BKD as described by Bruno (9).

INDIRECT FLUORESCENT ANTIBODY TEST

The preparation of the multi-well slides was done following the protocol outlined by Newbound et al (8,11). The first pair of wells were filled with approximately 20 μL of positive polyclonal. This serum had been collected from mice inoculated with anterior kidney and retrobublar tissue homogenate obtained from chinook salmon that had been infected either naturally or experimentally and that had been diagnosed with PL by histology (11). The serum was diluted to 1:500 with phosphate-buffered saline (PBS), pH 7.4. The serum inoculated wells represented the positive control and were used to demonstrate positive fluorescence. The second set of wells was filled with a similar volume of PBS and acted as the negative control to show a non-fluorescing field. The third and fourth sets of wells were filled with an IgG monoclonal and the fifth and sixth pairs of wells were filled with an IgM monoclonal antibody. Both the IgG and the IgM monoclonal antibodies were developed specifically for the diagnosis of PL. Each antibody is believed to bind to a different receptor; using both had been recommended to increase the chance of diagnosing a PL-positive fish (11). Slides were incubated in a dark, humidified chamber at 22°C for 30 min. The slides were rinsed and washed twice for 5 min in PBS. All of the wells were then filled with 20 μL of a rabbit anti-mouse IgG, IgA and IgM conjugated with fluorescein isothiocyanate (FITC) (Zymed, San Francisco, California, USA) at a 1:100 dilution, and a drop of Evan's blue dye (BDH, Dartmouth, Nova Scotia). They were then incubated in the dark, humidified chamber for 30 min. The FITC-conjugated antibody was then removed with two 5-minute washes of sodium carbonate buffer [4.2% $NaHCO_3$/5.3% Na_2CO_2 (4:1)]. Coverslips were mounted using FA Mounting media (pH 7.0, DIFCO Laboratories, Detroit Michigan, USA).

The slides were examined using fluorescence microscopy using the method outlined by Newbound et al

(8). In order to reduce the risk of misclassification bias, the slides were coded so as to blind the primary investigator to their origin. Control wells were examined first to evaluate proper antigen binding and to appraise the level of background fluorescence. If no fluorescence was evident in the positive control well, or if excessive background fluorescence was observed in the negative control well, the slide was rejected and the duplicate slide was stained and examined. From each of the remaining wells, 10 fields consisting of a uniform monolayer of cell impressions were selected and examined at 400× magnification. All fluorescing cells were counted and given a subjective score of 1, 2 or 3, corresponding to the intensity of fluorescence, with 1 indicating a low level of fluorescence and 3 indicating an intense degree of fluorescence. The scores from the 80 fields were summed. Those fish with IFAT values equal to or exceeding 21, the cut-off determined by Newbound et al (8), were given a positive PL diagnosis, whereas those below the cut-off were considered negative for PL.

COMPARISON OF IFAT TO HISTOLOGY

Histology results were compared to the dichotomized IFAT results using 2 × 2 tables. Data were initially stratified by farm and then by BKD status. The level of agreement between the 2 tests was measured by calculating Cohen's kappa (18,21). A kappa is used to measure agreement beyond chance between 2 tests. If a quadrant was found to have a zero outcome, a correction factor of 0.5 was added to all 4 quadrants before calculating kappa. A kappa value of zero indicates no agreement beyond that due to random chance while a kappa of 1 indicates complete agreement (18,19). For clinical purposes, a kappa value of greater than 0.6 indicates excellent agreement (19). Chi-square tests were used to determine whether there were significant differences between the kappa value of each stratum. If no significance was found a pooled kappa was calculated (18). A chi-square test was also performed to determine whether kappa was significantly different from zero, i.e. no agreement beyond chance (18). The level of significance was set at $P < 0.05$ for the chi-square tests.

TABLE I. Plasmacytoid leukemia (PL) diagnostic results from 2-year-old chinook salmon from 3 British Columbia farms, as determined by histology and an indirect immunofluorescent antibody test (IFAT)

		Histology			
		+	−	Total	
IFAT[abc]	+	23	27	50	Q = 0.28 (50/177)
	−	44	83	127	1-Q = 0.72 (127/177)
	Total	67	110	177	

[a] Fish with IFAT scores ≥ 21 were diagnosed as PL+
[b] Sensitivity of IFAT (relative to histology) = 0.34 (SE = 0.06); Quality index = 0.08
[c] Specificity of IFAT (relative to histology) = 0.75 (SE = 0.04); Quality index = 0.11

Sensitivity and specificity for the IFAT results relative to the histology results were calculated for each farm. Sensitivity described the proportion of samples diagnosed PL-positive with histology that were also positive with the IFAT. Specificity described the proportion of samples diagnosed PL-negative with histology that were also IFAT-negative (16,17). A quality index was calculated for each IFAT sensitivity and specificity using the method outlined by Kraemer (16). Sensitivity for a test that is random or better than random range from Q to 1, where Q is defined as the apparent prevalence of the disease in the tested population as estimated by the "new" test. In this study, Q was estimated by the proportion of fish that tested positive by IFAT. Specificity for a test that is random or better than random would range from 1-Q to 1 (16). Quality indices describe the position of sensitivity and specificity on this scale (Q to 1 and 1-Q to 1, respectively), thereby giving an indication of the test's legitimacy. A test whose results are not better than random would have quality index values of zero. Legitimate tests should have a quality index approaching one (16). A chi-square test was performed to determine whether the IFAT results were better than random (16), using a significance level of $P < 0.05$.

Sensitivity, specificity and their respective quality indices for the IFAT were calculated for 12 cut-off points other than 21 to determine whether a more appropriate cut-off point existed. The most suitable cut-off was determined to be the one with the highest sensitivity and specificity quality index weighted average (16).

RESULTS

All 3 farms visited were found to have 2-year-old chinook salmon exhibiting gross signs of PL. Since only moribund fish were sampled an estimate of on farm disease prevalence could not be made. One hundred sixty-two of the 177 fish collected for histology and IFAT were examined grossly by the primary investigator. Fifteen fish were not examined grossly, because they were not collected by the primary investigator. Of those fish examined grossly, 82% had pale gills indicating some degree of anemia, 75% had evidence of splenomegaly, 81% had evidence of renomegaly and 19% exhibited a degree of exophthalmia. Bacterial kidney disease was diagnosed by histology on 2 of the 3 farms.

Using histology, 67 (38%) of the 177 fish were diagnosed as having PL, with 3 of these exhibiting severe histological signs of PL. The IFAT, using the cut-off point of 21, found 50 (28%) of 177 fish to be positive for PL. Five of the duplicate slides had to be examined due to excessive background fluorescence on the original slide. The IFAT scores ranged from 0 to 270 with a mean of 26. Anterior kidney tissue had a far greater proportion of fluorescing cells than did spleen tissue. Differences in the numbers of fluorescing cells between the 2 MAbs were not examined. Cells of varying sizes were found to fluoresce with the IFAT, with fluorescence restricted to the cell wall.

Agreements between test results were not found differ significantly among the 3 farms, so a pooled farm kappa was calculated. Table I compares IFAT results to the histological findings for the pooled data. The pooled kappa value was calaculated to be 0.10 (SE = 0.07), well below the desired limit of 0.6, and was not significantly different from 0. This suggested that there was no agreement between the tests other than that due to chance alone. Also, the 3 fish exhibiting the most severe histological cases

of PL were diagnosed PL-negative with IFAT.

Sensitivity and specificity of the IFAT with their corresponding quality indices are shown, for the pooled farm data is shown in Table I. The sensitivity and specificity were only slightly higher than Q and 1-Q, respectively. Consequently both quality indices were very low, indicating that the IFAT was not a legitimate test for PL diagnosis. This was confirmed by the chi-square test ($\chi^2 = 1.55$), which indicated that the test was no better than random ($P < 0.05$).

When results were stratified by the presence of BKD, no significant difference was observed among the farms. Therefore a pooled kappa could be calculated. Table II presents histology and IFAT results further stratified by BKD status. Farm 3 showed no histological evidence of BKD. The pooled kappa for the BKD positive (based on results from farm 1 and 2) and the BKD negative (based on results from all 3 farms) strata was 0.04 (SE = 0.13) and 0.12 (SE = 0.08) respectively. Both kappa values fail to establish any agreement beyond that due to chance. Also, stratifying by BKD status did not affect sensitivity or specificity values of either farm 1 or 2.

Pooled data were used to evaluate 12 cut-off points for the IFAT ranging from 0 to 100. A cut-off of 45 provided the highest weighted kappa. This cut-off increased the specificity of the IFAT to, 0.91 with a quality index of 0.44, however at this cut-off the sensitivity dropped to 0.28, with a quality index of 0.14.

TABLE II. Plasmacytoid leukemia (PL) diagnostic results, categorized by bacterial kidney disease (BKD) status, from 2-year old chinook salmon from 3 British Columbia farms, as determined by histology and an indirect antibody immunofluorescent test (IFAT)

		Histology					
		BKD - Positive[acd]			BKD - Negative[ef]		
		+	−	Total	+	−	Total
IFAT[b]	+	2	11	13	21	16	37
	−	4	32	36	40	51	91
	Total	6	43	49	61	67	128

[a] Farms 1 and 2 only
[b] Fish with IFAT scores ≥ 21 were diagnosed as PL+
[c] Sensitivity of IFAT (relative to histology) = 0.33 (SE = 0.18)
[d] Specificity of IFAT (relative to histology) = 0.74 (SE = 0.08)
[e] Sensitivity of IFAT (relative to histology) = 0.34 (SE = 0.06)
[f] Specificity of IFAT (relative to histology) = 0.76 (SE = 0.05)

DISCUSSION

In a questionnaire survey, Stephen et al (4) found that PL was widely distributed in BC. Little is known, however, about risk factors for and consequences of PL, partly because of the lack of a relatively quick, sensitive and specific diagnostic test. The IFAT investigated in the present study was developed for this reason. Unfortunately, in contrast to earlier laboratory evaluations (8), the IFAT was shown to perform poorly in field situations.

Tissue imprints from both anterior kidney and spleen were used to evaluate the IFAT. The present study found more fluorescing cells in the kidney tissue than in the splenic tissue. This differs from the findings of Newbound (11), who found a higher proportion of fluorescing cells in splenic tissue. This may indicate that the distribution of antigen that binds to the IFAT MAbs differs in naturally and experimentally infected fish. The cells that fluoresced appeared to have fluorescence concentrated in the cell walls. These cells appeared similar in size distribution to those found by Newbound (11). The identity of these cell types was not identified in the present study.

Background fluorescence is a commonly encountered problem in fluorescent antibody tests (13). Cvitanich (20) determined that antibody incubations of greater than 60 min resulted in an increase in background fluorescence. In the present study, a 30-minute antibody incubation was used. Background fluorescence was a problem in less than 3% of the first run slides.

The true sensitivity and specificity of the IFAT could not be calculated in the field because there was no true gold standard diagnostic test with which to compare it (19). The IFAT was therefore evaluated against histology, the standard test being used at the time. Stephen et al (21) suggested that histology provided inconsistent diagnoses for PL. However, a high level of agreement (kappa = 0.70) was achieved when 3 of the present investigators independently evaluated histology, from 132 of the samples collected for the present study, using the criteria described above in the Materials and Methods (22). These same criteria were used by Newbound et al (8) in their experiments.

Gross evidence of PL along with positive histology and IFAT results were observed on all three farms participating in this study. However, even though the proportion of fish found positive by histology and by the IFAT did not differ greatly, the level of agreement between the 2 tests was very poor. Newbound et al (8) also had poor agreement between histology and IFAT in their laboratory experiments (kappa = 0.22). This brings into question the suitability of the present IFAT as a diagnostic tool for PL. The IFAT developers indicated that histology had very high specificity. Hence, unless the true prevalence is very low, a large proportion of the fish diagnosed with PL by histology are truly infected (19). Therefore, an estimate of the sensitivity of the IFAT relative to histology should be fairly accurate for those fish in which disease has progressed to the point of producing observable histological changes. In the present study, however, many of the histology diagnoses were not found to be positive with the IFAT, resulting in a low sensitivity estimate for the IFAT, substantially lower than that calculated with experimental infection (8). In addition, the quality index associated with the sensitivity was very low. Newbound et al (8) speculated that the MAbs used in the IFAT may be specific to antigens more common in subclinical or in very early stages of PL, before histological changes would become apparent, which could cause cases that test positive with histology to test negative with the IFAT. The major concern with this suggestion is rooted in the methods used to develop the IFAT. Previous researchers who have developed IFATs for fish diseases and found them to be useful

diagnostic tools, have used MAbs developed against specific pathogens (23–25). When the present IFAT for PL was developed, a causative pathogen had not been identified. Therefore, the test was developed using whole organs harvested from fish, not subclinically infected, but with clinical and histological signs of PL. This mixed homogenate of whole organ tissue would contain a multitude of different antigens, only some that are unique to PL. It is possible, therefore, that the low sensitivity of the present IFAT is because the MAbs are not specific to an antigen unique to PL. Unfortunately, the IFAT requires lethal sampling and it was not possible to investigate whether the IFAT was detecting subclinical PL. However, considering the types of tissues used in the development of the MAbs and the general poor performance in the field trial, the IFAT's ability to diagnose subclinical PL remains doubtful.

When the IFAT was developed, it was thought that it would be more effective in diagnosing PL in fish simultaneously infected with other diseases, such as BKD. In the laboratory evaluation, where 'inoculation status' represented the gold standard, a comparison of IFAT and histology in the presence of BKD indicated that IFAT was better at detecting PL than was histology (8). If the IFAT is better able to detect PL in the presence of BKD, then one would expect that a higher proportion of fish with concurrent BKD and PL infections would be detected as PL-positive by IFAT than histology. Therefore, the specificity of the IFAT, when measured against histology, would be lower in the group with BKD infection, as a result of a higher frequency of false negative diagnoses made by histology. In the present study, no significant differences were found in the specificity of the positive and negative BKD groups. Therefore, it cannot be concluded that the IFAT is better at detecting PL in the presence of BKD than histology.

It is possible that the development and expression of natural PL infections differ from experimentally induced infections. Experimentally induced infections generally involve higher loading doses, artificial routes of infection, and shorter disease duration. Since, the IFAT was developed to diagnose naturally occurring PL infections, it was deemed essential to evaluate it not only experimentally, but also in its natural setting. Unfortunately, the results of the field study indicated that the IFAT, in its present state, is not a useful diagnostic tool for PL. This study illustrates the importance of evaluating tests not only within the laboratory but also in the setting where their use is intended. Until a reliable diagnostic test for PL is developed, diagnosis will continue to be made based on gathering a good history, performing a thorough necropsy, and recognizing suspicious gross signs and histology. In cases where a mixed BKD/PL infection is suspected, a poorer than expected response to BKD therapy may be used to indirectly suggest a diagnosis.

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REFERENCES

1. **KENT ML, GROFF JM, TRAXLER GS, ZINKL JG, BAGSHAW JW.** Plasmacytoid leukemia in seawater reared chinook salmon *Oncorhynchus tshawytscha*. Dis Aquat Org 1990; 8: 199–209.
2. **NEWBOUND GC, KENT ML.** Plasmacytoid leukemia (Marine anemia): viral or microsporidian etiology. AFS-FHS Newsletter 1991; 19: 1–2.
3. **BRACKETT JB, NEWBOUND GC, SPEARE DJ.** A fall survey of saltwater morbidity and mortality of farmed salmon in British Columbia. Province of British Columbia, Ministry of Agriculture and Fisheries, Victoria, British Columbia, 1990.
4. **STEPHEN C, RIBBLE CS, KENT ML.** Descriptive epidemiology of marine anemia in seapen-reared salmon in southern Britsh Columbia. Can Vet J 1996; 37: 420–425.
5. **CHILMONCZYK S, COX WT, HEDRICK RP.** *Enterocytozoon salmonis* N sp: an intranuclear microsporidium from salmonid fish. J Protozool 1991; 38: 264–269.
6. **KENT ML, DAWE SC.** Further evidence for a viral etiology in plasmacytoid leukemia of chinook salmon *Oncorhynchus tshawytscha*. Dis Aquat Org 1993; 15: 115–121.
7. **KENT ML, POPPE TT.** Diseases of cold-water marine fish in cage culture. In: Woo PTK, Bruno DW, Lim SL, eds. Diseases of finfish in cage culture. Oxon: Agriculture and Biosciences Intl. Publ, 1998 (in press).
8. **NEWBOUND GC, MARKHAM RJF, KENT ML, SPEARE DJ, THORBURN M.** Comparative analysis of a monoclonal-antibody-based immunofluorescence test for plasmacytoid leukemia in chinook salmon *Oncorhynchus tshawytscha*. Dis Aquat Org 1995; 21: 123–130.
9. **BRUNO DW.** Histopathology of bacterial kidney disease in laboratory infected rainbow trout, *Salmo gairdneri* Richardson, and Atlantic Salmon, *Salmo salar* L with reference to naturally infected fish. J Fish Dis 1986; 9: 523–527.
10. **BRACKETT JB, NEWBOUND GC, SPEARE DJ.** A summer survey of saltwater morbidity and mortality of farmed salmon in British Columbia. Province of British Columbia, Ministry of Agriculture and Fisheries, Victoria, British Columbia, 1991.
11. **NEWBOUND GC, MARKHAM RJ, SPEARE DJ, et al.** Production of monoclonal antibodies specific for antigens derived from tissue of chinook salmon, *Oncorhynchus tschawytscha*, affected with plasmacytoid leukemia. Am J Vet Res 1993; 54: 1426–1431.
12. **ELLIOTT DG, BARILA TY.** Membrane filtration-fluorescent antibody staining procedure for detecting and quantifying *Renibacterium salmoninarum* in coelomic fluid of chinook salmon (*Oncorhynchus tshawytscha*). Can J Fish Aquat Sci 1987; 44: 206–210.
13. **LAPATRA SE, ROBERTI KA, ROHOVEC JS, FRYER JL.** Fluorescent antibody test for the rapid diagnosis of infectious hematopoietic necrosis. J Aquat Anim H 1989; 1: 29–36.
14. **HSU H, BOWSER PR, SCHACHTE JH.** Development and evaluation of a monoclonal-antibody-based enzyme-linked immunosorbent assay for the diagnosis of *Renibacterium salmoninarum*. J Aquat Anim H 1991; 3: 68–175.
15. **STEPHEN C, RIBBLE CS.** Epidemiology of marine anemia (plasmacytoid leukemia) in farmed salmon of British Columbia. British Columbia Ministry of Agriculture, Fisheries and Food, Victoria, British Columbia, 1993.
16. **KRAEMER HC.** Evaluating medical tests: Objective and quantitative guidelines. Sage Publications, 1992.
17. **HUMANSON GL.** Animal tissue techniques. W.H. Freeman and Co., 1979.
18. **FLEISS JL.** Statistical methods for rates and proportions. John Wiley and Sons, 1981.
19. **MARTIN SW, BONNETT B.** Clinical epidemiology. Can Vet J 1987; 28: 318–325.
20. **CVITANICH JD.** Improvements in the direct fluorescent antibody technique for the detection, identification, and quantification

of Renibacterium salmoninarum in salmonid kidney smears. J Aquat Ani H 1994; 6: 1–12.
21. **STEPHEN C, RIBBLE CS, KENT ML.** Observer variation in the histologic diagnosis of plasmacytoid leukemia (marine anemia). Can J Vet Res 1985; 59: 15–19.
22. **SAKSIDA SM.** Evaluation of an immunofluorescent antibody test for diagnosis of plasmacytoid leukemia in chinook salmon (MSc thesis). Guelph, Ontario, University of Guelph, 1995: 79.
23. **BULLOCK GL, GRIFFIN BR, STUCKEY HM.** Detection of *Corynebacterium salmoninus* by direct fluorescent antibody test. Can J Fish Aquat Sci 1980; 7: 719–721.
24. **SAKAI M, ATSUTA S, KOBAYASHI M.** Comparison of methods used to detect *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease. J Aquat Ani H 1989; 1: 21–24.
25. **LANNAN CN, EWING SA, FRYER JL.** A fluorescent antibody test for detection of the rickettsia causing disease in chilean salmonids. J Aquat Ani H 1991; 4: 229–234.